A Multivalent Minigene Vaccine, Containing B-Cell, Cytotoxic T-Lymphocyte, and T_h Epitopes from Several Microbes, Induces Appropriate Responses In Vivo and Confers Protection against More than One Pathogen†

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Received 30 September 1996/Accepted 5 December 1996

The development of safe and effective vaccines remains a major goal in the prevention, and perhaps treatment, of infectious diseases. Ideally, a single vaccine would confer protection against several pathogens and would induce both cellular and humoral arms of the immune response. We originally demonstrated that two virus-specific cytotoxic T-lymphocyte (CTL) epitopes, from the same virus but presented by different major histocompatibility complex alleles, when linked in tandem as minigenes in a recombinant vaccinia virus, could confer complete protection against subsequent viral challenge. In the study, we extended this approach, which we termed string of beads, expanding the immunogenic scope in two ways: first, by introduction of T helper (T_h) and B-cell (antibody) epitopes alongside CTL epitopes and second, by including immunogenic sequences from a variety of infectious agents, five viruses and one bacterium. The vaccine (VV-sv) comprises CTL epitopes from Sendai virus, respiratory syncytial virus, and lymphocytic choriomeningitis virus (LCMV); T_h epitopes from vesicular stomatitis virus and *Mycobacterium tuberculosis*; and an antibody epitope from mengovirus. The construct contains a single start codon, and the epitopes are linked directly, without intervening spacer amino acids. There was some concern that the combination of several normally immunodominant epitopes might result in a new hierarchy of dominance, in which certain epitopes predominated and others exhibited reduced immunogenicity. However we show that when analyzed in tissue culture and in vivo, all six epitopes are expressed. CTL and T_h cells are induced in vivo, along with neutralizing antibody. The induced immunity is biologically relevant: after VV-sv immunization, the antimengovirus antibody confers protection against mengovirus challenge. Similarly, CTL induced by the LCMV epitope protected mice against challenge with this agent. Thus, a polyvalent, minigene-based vaccine can simultaneously induce several classes of immune response and thereby can confer protection against diverse pathogens.

Antiviral vaccines have been signally successful, allowing worldwide eradication of smallpox and, at least in the developed countries, the approaching eradication of poliomyelitis. Both antibodies and CD8⁺ cytotoxic T lymphocytes (CTL) are important in combating primary virus infections and in vaccine-induced antiviral immunity (reviewed in reference 37).

For any one virus, there are frequently only a few immunodominant epitopes of biological relevance. It is against these epitopes that the protective immune response is mounted. We have previously shown that isolated CTL epitopes can, when expressed in a recombinant vaccinia virus, confer protective immunity against a normally lethal challenge dose of virus (20) and can confer limited protection against certain bacterial pathogens (4). Furthermore, in a concept we termed a string of beads (39), we have shown that two epitopes, each presented on a different major histocompatibility complex (MHC) class I background, can be linked in tandem to confer protection upon mice of both MHC haplotypes. In the present study, we expand the immunological scope of our previous studies in two ways. First, in addition to CTL epitopes, we have included sequences encoding antibody (B cell) and T helper (T_h) cell epitopes, to establish whether the latter two antigen-specific

immune responses could, like CTL responses, be induced by minigene expression. Second, since an ideal vaccine would confer protection against several pathogens, we have encoded epitopes from a variety of pathogens. Several of these epitopes are immunodominant in the wild-type pathogen, and some have the same presenting MHC allele. We were concerned that the combination of several immunodominant epitopes might lead to suppression of presentation of some epitopes, while others were favored. This study affords us the opportunity to address this question.

We have selected defined dominant epitopes from vesicular stomatitis virus (VSV) (7), respiratory syncytial virus (RSV) (22), mengovirus (24), Sendai virus (SV) (9), and lymphocytic choriomeningitis virus (LCMV) (20) and a promiscuous epitope, immunogenic on several MHC backgrounds, from the bacterium *Mycobacterium tuberculosis* (MTB epitope) (33). These six epitopes have been fused together, with a single start codon and no intervening amino acids. Two stop codons are present at the end of the construct. We show that (i) most epitopes are expressed in tissue culture and are recognized appropriately; (ii) the sequences induce epitope-specific, MHC-restricted immune responses in vivo; and (iii) the immunity induced protects against subsequent challenge by at least two of the selected viruses. These studies therefore show that immunodominant epitopes from heterogeneous pathogens can be grouped together without overt loss of immunogenicity for any epitope, that minigenes can induce not only CTL responses but also T_h and antibody responses, and that a single

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† Publication no. 10209-NP from the Scripps Research Institute.

1	ATG	TCT	TCT	AAA	GCT	CAA	GTT	TTT	GAA	CAT	CCT
	M	S	S	K	A	Q	V	F	E	H	P
34	CAT	ATT	CAA	GAT	GCT	GCT	TCT	CAA	TTA	TCT	TAT
	H	I	Q	D	A	A	S	Q	L	S	Y
67	ATT	GGT	TCT	ATT	AAT	AAT	ATT	GAT	CAA	GTT	CAT
	I	G	S	I	N	N	I	D	Q	V	H
100	TTT	CAA	CCT	TTA	CCT	CCT	GCT	GTT	GTT	AAA	TTA
	F	Q	P	L	P	P	A	V	V	K	L
133	TCT	GAT	GCT	TTA	ATT	CCT	ACT	TCT	GGT	GAT	AAA
	S	D	A	L	I	P	T	S	G	D	K
166	ATT	GAT	ATG	ACT	CCT	CGT	GCT	GGT	GTT	TTA	ATG
	I	D	M	T	P	R	A	G	V	L	M
199	TTA	GAA	TTT	GCT	CCT	GGT	AAT	TAT	CCT	GCT	TTA
	L	E	F	A	P	G	N	Y	P	A	L
232	AAA	GCT	GTT	TAT	AAT	TTT	GCT	ACT	TGT	GGT	ATT
	K	A	V	Y	N	F	A	T	C	G	I

FIG. 1. Sequences of multiple minigenes. The minigenes of dominant epitopes from different viruses and bacteria were fused as shown. There is an ATG at the beginning of the construct. The minigenes, distinguished by blocks of shading, were arranged in the order VSV, RSV, *M. tuberculosis*, mengovirus, SV, and LCMV.

vaccine which induces protective immunity to more than one pathogen can be designed, based on minigenes.

MATERIALS AND METHODS

Viruses. Vaccinia virus was propagated in HeLa cells and was titrated as reported previously (36). LCMV Armstrong strain was used (14). The mengovirus M plaque-type variant was a kind gift from D. G. Scraba, University of Alberta, Edmonton, Alberta, Canada.

Construction of recombinant vaccinia virus carrying the multiple minigenes. The sequence of the multivalent minigene construct is shown in Fig. 1. Oligonucleotides encoding GP₄₁₅₋₄₃₃ from VSV Indiana, M₂₈₂₋₉₀ from RSV, P₃₅₀₋₃₆₉ from *M. tuberculosis*, VP₁₂₅₉₋₂₇₇ from mengovirus, NP₃₂₄₋₃₃₂ from SV, and GP₁₃₃₋₄₃ from LCMV were synthesized (Gibco, Gaithersburg, Md.) in the above order as 60-mers with 20 bases overlapping. No spacer oligonucleotides were placed between each epitope. The synthesized 60-mers were stepwise fused and amplified by PCR. The program was 30 cycles of 94°C for 10 s, 45°C for 20 s, and 72°C for 20 s, using *PFU* DNA polymerase (Stratagene, San Diego, Calif.). The final product, whose sequence is shown in Fig. 1, was prepared for cloning, and fitted with two in-frame stop codons, by a final PCR using primer 5'sv (CCC GCC GGC CAT GTC TTC TAA AGC TCA AGT), which contains a *NaeI* site, and primer 3'sv (CCC CGC GGC CGC TAT TAA ATA CCA CAA GTA GCA), which contains a *NotI* site and two stop codons in complementary orientation (5'CTATTA3' becomes 5'TAATAG3' in the sense strand). The final PCR product was cleaved with *NaeI* and *NotI* and cloned into a vaccinia virus transfer plasmid, which was then introduced into vaccinia virus by standard methods. The flowchart of the construction is shown in Fig. 2. DNA from the recombinant vaccinia virus with multiple minigenes was PCR amplified and sequenced (29) to confirm correctness and was designated as VV-sv; a control recombinant vaccinia virus, identical to VV-sv but lacking the multiple minigenes, is named VV-SC11.

Peptides and T-cell hybridomas. Peptide M₂₈₂₋₉₀ of RSV and GP₁₃₃₋₄₃ of LCMV were synthesized in the Core Facility of The Scripps Research Institute. Peptide VP₁₂₅₉₋₂₇₇ of mengovirus was a gift from James Bittle of the same institute. T-cell hybridoma 31.2#10, specific to VSV Indiana GP₄₁₅₋₄₃₃, was given by Hans Hengartner, University of Zurich. The clone was maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10⁻⁵ M β-mercaptoethanol. T-cell hybridoma B3.4G6, specific to NP₃₂₄₋₃₃₂ of SV (Enders strain), the gift of Geoffrey A. Cole (St. Jude Children's Research Hospital), was maintained in RPMI 1640 supplemented as described above. T-cell hybridoma BC.H20, specific to P₃₅₀₋₃₆₉ of *M. tuberculosis*, and the P₃₅₀₋₃₆₉ peptide were provided by H. Martin Vordermeier, MRC Clinical Science Centre, London, England. These cells were grown in RPMI 1640 with 10% FCS, 100 U of penicillin per ml, 100 µg of streptomycin per ml, 10⁻⁵ M β-mercaptoethanol, and hypoxanthine-thymidine (HT) (Boehringer Mannheim, Indianapolis, Ind.). A summary of the epitopes and the related hybridomas is shown in Table 1.

Immunization of mice. BALB/cByJ and MC57BL/6 mice from the Scripps facility were used when 8 to 12 weeks old. Mice were inoculated intraperitoneally (i.p.) with 10⁷ PFU of VV-sv or VV-SC11 in saline. For induction of antisera to the mengovirus sequence, the mice were boosted with 5 × 10⁶ PFU of VV-sv per mouse 2 weeks after the first immunization.

6 x 60mer overlapping oligonucleotides

FIG. 2. Construction of VV-sv. 60-mer oligonucleotides with 20-base overlaps were synthesized and stepwise fused together by PCR. The complete insert was obtained by amplification with two primers containing sequences complementary to both ends of the final fusion product and the restriction sites *NaeI* and *NotI*. Cloning and introduction into recombinant vaccinia virus were carried out as described in the text. MCS, multiple cloning site; Amp-r, ampicillin resistance; beta-gal, β-galactosidase.

Use of T-cell hybridomas to detect minigene expression in tissue culture. Expression of three of the selected epitopes can be detected by using antigen-specific MHC-restricted T-cell hybridomas, activation of which leads to interleukin-2 (IL-2) secretion. Splenic lymphocytes from either BALB/cByJ (*H-2^d*) or C57BL/6 (*H-2^b*) mice were used as antigen-presenting cells. Spleens were taken from mice and disrupted in a glass homogenizer. The erythrocytes (RBC) were lysed with 0.83% NH₄Cl, and the residue was washed three times with complete RPMI 1640 medium (supplemented with 10% FCS, 100 U of penicillin, and 100 µg of streptomycin). The splenocytes were then infected at a multiplicity of infection of 5 with either VV-sv or VV-SC11 and incubated for 4 h at 37°C in a 5% CO₂ incubator. When peptide antigen (the MTB epitope) was used to

TABLE 1. Minigenes used

Virus or bacterium	Epitope	Type	Specific T-cell hybridoma	MHC allele(s) or cell type
VSV	GP ₄₁₅₋₄₃₃	Helper	31.2#10	<i>I-A^b</i>
Mengo virus	VP ₁₂₅₉₋₂₇₇	Antibody		B cell
RSV	M ₂₈₂₋₉₀	CTL		<i>K^d</i>
<i>M. tuberculosis</i>	P ₃₅₀₋₃₆₉	Helper	BC.H20	<i>I-A^b</i> and <i>I-A^d</i>
SV	NP ₃₂₄₋₃₃₂	CTL	B3.4G6	<i>K^b</i>
LCMV	GP ₁₃₃₋₄₃	CTL		<i>D^b</i>

stimulate a T-cell hybridoma, the splenocytes were incubated with peptide (50 or 350 µg/ml; see the legend to Fig. 4) for 1.5 h at 37°C. The infected or peptide-coated cells were then washed twice, irradiated (3,000 rads), and plated at 3×10^5 cells/well in a flat-bottom 96-well plate. The T-cell hybridomas were added (5×10^4 to 1×10^5 cells in 100 µl per well) in triplicate. After incubation for 36 to 48 h, the supernatants were taken from each well, and IL-2 levels were determined by enzyme-linked immunosorbent assay (ELISA).

ELISA for detection of IL-2 and serum antibody. ELISA was used to detect IL-2 in the supernatants of the T-cell hybridoma experiments and the levels of antibody against mengovirus. For the IL-2 assay, goat anti-mouse IL-2 immunoglobulin G (IgG) (Pharmingen, San Diego, Calif.) was diluted in a coating buffer (0.1 M NaHCO₃ [pH 8.2]) to 2 µg/ml. Fifty microliters of diluted antigen was added to each well of a 96-well plate (Falcon, Oxnard, Calif.), which was incubated at 4°C overnight, washed three times with 0.05% Tween 20-phosphate-buffered saline (PBS), blocked with 10% FCS-PBS for 2 h at room temperature, and washed twice. Fifty microliters of supernatant from individual wells of the T-cell hybridoma plate (prepared as described above) was added in duplicate and incubated for 4 h at room temperature. Mouse recombinant IL-2 was used in serial dilutions from 15 to 1,000 ng/ml as a standard. The plate was washed three times, and 100 µl of biotinylated goat antimouse IL-2 IgG (1 µg/ml) was added. After 45 min of incubation, the plate was washed three times, 100 µl of a 1:5,000 dilution of avidin-peroxidase (Boehringer Mannheim) was added, and the mixture was incubated for 30 min. After washing, peroxidase substrate *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, Mo.) was added, and the reaction was stopped after 10 min by addition of 50 µl of 3 M HCl. Absorption at 492 nm was determined by using a Flow TiterTek (ICN, Costa Mesa, Calif.).

To determine levels of antibody to mengovirus, the mengovirus VP1₂₅₉₋₂₇₇ peptide was used as an antigen. The protocol was basically as described above, with the following exceptions. Wells were coated overnight with 100 µl of the diluted peptide (1 µg/ml). The incubation time for sera was 2 h, and detection of bound antibody was carried out with biotinylated goat anti-(mouse IgG) IgG (Vector, Burlingame, Calif.) at a 1:200 dilution.

T-cell proliferation assay to identify cells responding to the promiscuous MTB epitope. Six weeks after i.p. immunization with either VV-sv, VV-SC11, LCMV, or saline, effector cells were prepared. Mice spleens were taken and homogenized. After lysing of RBC, splenocytes from two mice of each group were passed through a nylon wool column, and T cells were collected (9a). Stimulator cells, prepared from either normal BALB/cByJ or C57BL/6 mice spleen, were incubated with the promiscuous peptide P₃₅₀₋₃₆₉ from *M. tuberculosis* (50 µg/ml for BALB/c and 350 µg/ml for C57) at 37°C for 1.5 h, washed, and irradiated (3,000 rads). The irradiated stimulator cells were then plated at 5×10^5 cells/well in a 96-well plate. The partially purified T cells (in numbers ranging from 50,000 to 400,000/well) were then added in triplicate and incubated for 4 days. On day 4, 1 µCi of [³H]methylthymidine (Amersham, Arlington Heights, Ill.) was added to each well; 4 h later, cells were harvested and counts incorporated were determined.

In vitro T-cell cytotoxicity assay. Target cells were BALB/c17 (*H-2^d*) or MC57 (*H-2^b*). Cells (10^6 /ml) were incubated with the specified peptides at 50 to 100 µg/ml in 1 ml of complete RPMI 1640 medium with 100 µCi of ⁵¹Cr. After incubation at 37°C for 1.5 h, the target cells were washed three times with RPMI 1640 medium. Where virus-infected target cells were used, cells were infected (multiplicity of infection of 3) 48 h before assay (LCMV) or 18 h before assay (vaccinia virus). CTL were prepared from mouse spleens harvested at the times specified in the text. Spleens were disrupted, RBC were lysed, and splenocytes were incubated in triplicate wells with target cells for 5 h. From each well, 100 µl of supernatant was harvested, and radioactivity was counted in a IsoData γ counter. Percentage specific ⁵¹Cr release was calculated as (sample release cpm – spontaneous release cpm) × 100/(total release cpm – spontaneous release cpm). When spleens were to be used as a source of CTL, and also were to be titrated for the presence of LCMV (see below), each spleen was bisected; the halves to be used as CTL sources were pooled, while the remaining halves were retained, each to be titrated separately.

Evaluation of protective efficacy of the VV-sv vaccine. (i) Protection against LCMV. Mice were immunized by the i.p. route either with saline (negative control), LCMV (2×10^5 PFU; positive control), or recombinant vaccinia virus (VV-sv or VV-SC11, 10^7 PFU i.p.). Six weeks later, mice were challenged with 2×10^5 PFU of LCMV i.p. Four days after challenge, spleens were taken and bisected. Half of each spleen was analyzed for LCMV titers (see below) to determine the extent to which previous immunization had enhanced the ability of the mouse to control virus replication. The other half of each spleen was used in a CTL assay as described above, to determine whether prior immunization had enhanced the anti-LCMV CTL response.

(ii) Protection against mengovirus. Mice were immunized either with recombinant vaccinia virus (VV-sv or VV-SC11, 10^7 PFU i.p.) and boosted after 2 weeks or with mengovirus (10^5 PFU i.p.), and 6 weeks later they were challenged with mengovirus (5×10^4 PFU i.p.). At 4 and 10 days postchallenge, mice were sacrificed, their brains were harvested, and virus titers were determined.

LCMV plaque assay. The method was the same as reported previously (41). Briefly, half of the spleen from each mouse was weighed and homogenized in 1 ml of complete Dulbecco modified Eagle medium (DMEM). Tenfold serial dilutions were made, and 330 µl was used to infect Vero76 cells. After 1 h, the inoculum was replaced by an agarose (1× DMEM, 0.5% sterile agarose), and the

plates were incubated at 37°C with 5% CO₂ for 4 days. Two milliliters of 25% formaldehyde was added to each well, and after a 1-h fixation, the overlays were removed and the cells were stained with 0.1% crystal violet.

Mengovirus plaque assay. Brains were harvested, snap-frozen in liquid N₂, and stored at –70°C before titration. On the day of titration, brains were weighted and disrupted by a tissue homogenizer in complete PBS. The brain homogenates were 10× serially diluted and plated on HeLa cells in six-well plates (200 µl per well). Following incubation at room temperature for 1 h with intermittent agitation, 4 ml of overlay medium (2× DMEM, 1.6% agar [1:1]) was added to each well. Plates were incubated at 37°C for 48 h and fixed with 25% formaldehyde for 45 min, and the overlay was removed. Plates were rinsed once with H₂O and stained with 0.1% crystal violet, and plaques were counted.

Statistical analyses. Statistical significance was determined by using Student's *t* test included in the SigmaStat program (Jandel Scientific software).

Virus neutralization assay. Preimmune and immune sera were collected from VV-sv and VV-SC11 immunized mice and heated at 56°C for 15 min. Twofold serial dilutions were made, and triplicate 50-µl samples were applied to a sterile 96-well flat-bottom plate; 10 50% tissue culture infective doses mengovirus was added to each well, and the materials were incubated for 1 h at 37°C. Indicator cells (2×10^4 HeLa cells) were added to each well, and the plate was incubated at 37°C with 5% CO₂ for 48 h. The supernatants were removed, and the wells were stained with 50 µl of 0.1% crystal violet.

RESULTS

The sequences of the epitopes are shown in Fig. 1, and information regarding the type of epitope, the MHC allele which presents it, and (where relevant) the T-cell hybridoma used to detect it is shown in Table 1.

CTL from LCMV-immunized mice recognize VV-sv-infected target cells in a virus-specific, MHC-restricted manner. An in vitro cytotoxicity assay was used to determine whether VV-sv correctly presented the LCMV CTL epitope on the surface of infected target cells. CTL were obtained from C57BL/6 mice and from BALB/c mice 7 days following LCMV infection. These cells were incubated with MC57 or BALB/c17 target cells; the target cells were either uninfected or infected with VV-SC11 or VV-sv. As shown in Fig. 3, high levels of specific lysis of VV-sv-infected cells were seen only when CTL from C57BL/6 mice were used and only with MC57 target cells (Fig. 3A). Specificity for the minigene sequence is demonstrated by the lack of lysis of VV-SC11-infected targets (Fig. 3A). MHC specificity is shown in the remaining panels.

Use of T-cell hybridomas to evaluate expression of VSV, SV, and MTB epitopes. C57BL/6 (*H-2^b*) and BALB/c17 (*H-2^d*) spleen cells were infected with VV-sv or VV-SC11 or were left uninfected. Four hours postinfection, the cells were irradiated and incubated with T-cell hybridomas specific for VSV (hybridoma 31.2#10), SV (hybridoma B3.4G6), or *M. tuberculosis* (hybridoma BC.H20). These cells secrete IL-2 in response to MHC-restricted antigen-specific stimulation. Thirty-six or 48 h later, the supernatants were harvested and IL-2 levels were determined by ELISA. T-cell hybridomas 31.2#10 (Fig. 4A) and B3.4G6 (Fig. 4B) responded specifically to VV-sv, and only on the appropriate (*H-2^b*) MHC background. These data indicate that the VSV and SV epitopes are appropriately presented on the surface of VV-sv-infected cells by *H-2^b* MHC class II and class I molecules, respectively. In contrast, no evidence of expression of the MTB epitope was apparent. This promiscuous epitope should have been presented by both *H-2^b* and *H-2^d* haplotypes (33), but hybridoma BC.H20 failed to respond to VV-sv-infected cells of either haplotype (Fig. 4C and D). To confirm the activity of BC.H20, peptide-coated cells were used; a strong and dose-dependent response was seen on the *H-2^d* background (Fig. 4C and D), but there was minimal response on the *H-2^b* haplotype. A stronger response on *H-2^d* than *H-2^b* has been previously observed (33), but the peptide concentrations used in our studies are similar to those used in previous work, in which responses were detected on the

FIG. 3. VV-sv expresses the LCMV CTL epitope GP1₃₃₋₄₃* MC57 ($H-2^b$) (A and B) or BALB/c17 (C and D) cells were infected with VV-sv, VV-SC11, or LCMV and used as targets in an in vitro cytotoxicity assay. Effector cells were taken 7 days postinfection (with 2×10^5 LCMV) from C57BL/6 (A and D) or BALB/c (B and C) mice and were incubated for 5 h with target cells at the effector/target (E/T) ratios shown on the x axis.

$H-2^b$ background; the reason for this lack of $H-2^b$ -restricted response is unclear.

VV-sv immunization produces T cells responsive to the MTB epitope. Since epitopes located before (VSV) and after (SV and LCMV) the MTB epitope were appropriately expressed in tissue culture, it was highly likely that the promiscuous epitope sequence was being translated in VV-sv-infected cells. However, the failed recognition by clone BC.H20 suggested that the normal sequence was not being presented by the appropriate class II molecules. We hypothesized that the epitope might be processed differently when embedded in the minigene construct and that a related, alternately processed

form of the sequence, although not recognizable by the T-cell hybridoma, would nevertheless induce T-cell responses in vivo. Mice of both haplotypes were therefore immunized with VV-sv, VV-SC11, or saline; 6 weeks later, splenocytes were taken, and T were cells partially purified with nylon wool and incubated with the MTB P₃₅₀₋₃₆₉ peptide-coated irradiated spleen cells. After 4 days of this secondary restimulation, a thymidine uptake assay was carried out. As shown in Fig. 5A, T cells from VV-sv-infected $H-2^d$ mice showed markedly enhanced stimulation in response to this peptide. No such result was observed for $H-2^b$ mice (Fig. 5B). These data suggest that this epitope, when embedded in the collection of minigenes, can be pro-

FIG. 4. T-cell hybridomas detect expression of CTL and T_H epitope minigenes by VV-sv. Antigen-presenting cells were BALB/cByJ or C57BL/6 splenocytes infected either with VV-sv or VV-SC11, mock infected (nil), or peptide coated. T-cell hybridomas of the indicated specificities were incubated with these stimulator cells as described in Materials and Methods, and the IL-2 released from activated T-cell hybridomas was measured by ELISA. The IL-2 concentration shown is the mean of duplicate samples. To confirm the function of hybridoma BC.H20, the MTB epitope peptide, at 50 (C) or 350 (D) μ g/ml, was used to coat stimulator cells (see text).

cessed and presented in a form recognizable to some H-2^d T cells, but not to BC.H20, and that at least some of these T cells are able also to recognize (and therefore to be stimulated by) the standard peptide. Thus, we argue that the fine specificity of

the T-cell receptor on hybridoma BC.H20 allows recognition of the peptide but not of the epitope processed from the minigene construct, while the fine specificity of the T-cell receptor on the VV-sv-induced T cells allows recognition of both

FIG. 5. VV-sv induces T cells responsive to the promiscuous epitope from *M. tuberculosis*. Six weeks after immunization with a single dose of VV-sv or VV-SC11, T cells were isolated from BALB/cByJ (A) or C57BL/6 (B) mice, partially purified by nylon wool, and stimulated with peptide (P₃₅₀₋₃₆₉)-coated splenic cells. [³H]thymidine uptake was measured 4 days later. Values are means of triplicate samples.

moieties. We have previously demonstrated that CTL induced by similar sequences may show nonreciprocal or unidirectional cross-recognition (40).

VV-sv induces neutralizing antibodies to mengovirus. Pre-immune sera were drawn from 10 BALB/c mice, which were then immunized either with VV-sv (6 mice) or VV-SC11 (4 mice). Two weeks following immunization, sera were drawn from all 10 mice and evaluated in an ELISA for specificity against mengovirus. As shown in Fig. 6A, following a single immunization, five of the six VV-sv-immunized mice had elevated responses compared with mice receiving VV-SC11. Immediately after these serum samples were taken, all mice were boosted with the original virus (VV-sv or VV-SC11). Two weeks later, another serum sample was taken from each mouse and evaluated by ELISA. As shown in Fig. 6B, all six VV-sv-boosted animals showed enhanced responses to mengovirus.

The sera were pooled, heat inactivated, and mixed with 10 50% tissue culture infective doses of mengovirus. Following incubation for 1 h, the samples were tested for infectivity as described in Materials and Methods. Neutralization was seen only in sera from VV-sv-immunized mice, to a titer of approximately 1:10 (data not shown). The titer of positive control antisera from mengovirus-infected mice was 1:320.

VV-sv immunization induces CTLs specific to RSV and LCMV. To detect CTL activity against RSV, C57BL/6 and BALB/c mice were infected with VV-sv or VV-SC11; 7 days later, splenic lymphocytes were recovered and used directly (without restimulation) in an in vitro cytotoxicity assay using as target cells BALB/c17 (*H-2^d*) or MC57 (*H-2^b*) cells coated with the RSV peptide or with an unrelated peptide (NP peptide from LCMV). As shown in Fig. 7A, BALB cells coated with the RSV peptide were lysed by CTL from a VV-sv-infected BALB mouse. This reaction was MHC specific, as peptide-coated MC57 target cells were not lysed (data not shown). In addition, the reaction is peptide specific, as BALB target cells

coated with an unrelated peptide were not lysed by VV-sv-infected CTL to an extent greater than CTL from a VV-SC11-infected mouse (Fig. 7B). Note that this lytic activity was found in nonrestimulated CTL 7 days following infection with a recombinant vaccinia virus. It is unusual to detect such activity in nonrestimulated cells, but a prior report of activity induced by the same RSV epitope is consistent with our findings (22).

Splenocytes taken 7 days following infection with VV-sv, in the absence of restimulation, showed no activity against LCMV-infected target cells (not shown). Although this finding contrasts with those for the RSV epitope, it is consistent with all of our earlier studies and those of other laboratories, which show that following immunization with recombinant vaccinia viruses, a secondary stimulation is required to unveil LCMV-specific CTL. Therefore, *H-2^b* mice were immunized with VV-sv, VV-SC11, LCMV, or saline and were infected 6 weeks later with LCMV, to allow antigen-specific stimulation to occur in vivo. Spleens were harvested 4 days later, at which time point anti-LCMV CTL are low in previously naive animals and higher in previously primed animals; spleens were bisected, and pooled samples were used in a CTL assay as described in Materials and Methods. As shown in Fig. 7C, low levels of LCMV-specific lysis are detected in mice originally inoculated either with VV-SC11 or with saline; this reflects the developing anti-LCMV CTL activity at 4 days postchallenge in these previously unprimed mice. However, anti-LCMV CTL activity is greatly enhanced in mice previously immunized with VV-sv, reflecting the LCMV-specific priming of these mice, which leads to an accelerated response to LCMV challenge. Lysis was epitope specific, as cells coated with the LCMV GP/H2^b peptide, but not cells coated with the LCMV NP/L^d peptide, were lysed. CTL activity, as well as being epitope specific, was MHC restricted; as shown in Fig. 7D, LCMV-infected or peptide-coated BALB/c (*H-2^d*) target cells were not recognized by the MC57 (*H-2^b*) CTL.

FIG. 6. VV-sv elicits antibodies against mengovirus VP1₂₅₉₋₂₇₇ in mice. Two groups of BALB/cByJ mice were immunized i.p. with 10^7 PFU of either VV-sv ($n = 6$) or a control recombinant, VV-SC11 ($n = 4$). Antibody levels against mengovirus VP1₂₅₉₋₂₇₇ peptide were determined in individual mice by ELISA after a single immunization (A) and after a boost (B). The absorbances (optical densities at 492 nm [OD₄₉₂]) are the means of duplicates.

VV-sv immunization protects mice against subsequent challenge by LCMV or mengovirus. The CTL induced by VV-sv were biologically effective, as determined by reduction in LCMV titers in the VV-sv-immunized animals. As shown in Fig. 8A, all eight mice immunized with VV-SC11 had LCMV titers in the range of 5×10^3 to 5×10^5 PFU per g of spleen, similar to those in mice immunized with saline. In contrast, five of eight mice immunized with VV-sv had undetectable levels of LCMV. The average viral titers in these groups, and the statistical significance of each of the three virus-immunized groups compared to the saline-immunized control group, are shown in Fig. 8B. The protective effect of VV-sv immunization is statistically significant compared to saline ($P = 7.5 \times 10^{-4}$);

when directly compared to VV-SC11 immunization, VV-sv immunization remains highly significant ($P = 0.03$; not shown in Fig. 8). Furthermore, as shown in Fig. 8C, protection is conferred against mengovirus. Four days after challenge with this agent, mengovirus was undetectable in 3 of 10 VV-sv-immunized mice, and this group had, on average, 3 logs less virus in the brain than did VV-SC11-immunized control mice. By 10 days postchallenge, the average difference in titer increased to approximately 5 logs, and virus was undetectable in all VV-sv mice ($n = 6$), while all VV-SC11 vaccinees ($n = 4$) had readily detectable levels of virus. Thus, the data in Fig. 8 show that inoculation of VV-sv induces biologically significant CTL and antibody responses, capable of protecting against LCMV and mengovirus, respectively.

DISCUSSION

Studies from our own (20, 21) and other (1, 2, 27) laboratories demonstrated the importance of CTL in vaccine-induced antiviral protection; our approach was to design vaccines containing only CTL epitopes and resulted in the concept of epitope linkage in a multivalent vaccine which we termed a string-of-beads (25, 35, 39). In this study, we describe the construction and analysis of a recombinant vaccinia virus containing multiple minigenes, consisting of six sequences, five viral and one bacterial, comprising CTL, T_H, or antibody epitopes. Our results clearly show that a number of immunodominant peptide sequences can be coexpressed as a multivalent fusion protein and nevertheless can be efficiently presented on the surface of infected cells. This presentation allows not only recognition of recombinant-infected target cells but also in vivo induction of immune responses. VV-sv induces antibodies against mengovirus, MHC class II-restricted T-cell responses to the MTB epitope, and CTL responses to the RSV and LCMV CTL epitopes. The antibody response can neutralize mengovirus in vitro and protects against in vivo challenge; and the anti-LCMV CTL response also is protective.

Presentation of an epitope by an MHC class I molecule most often occurs when an endogenously synthesized protein is broken down by the proteasome, a multicomponent catalytic complex of cytosolic proteins (13). The resulting peptides are transported into the endoplasmic reticulum (ER) by TAP (transporters associated with antigen processing) proteins, where they associate with class I MHC molecules and β_2 -microglobulin; this trimolecular complex is subsequently transported to the cell surface. The cleavage specificity of the proteasome complex is still poorly understood. Some studies indicate preferential cleavage at certain amino acids (15), while other studies fail to support this notion (12). We have made no attempt to identify the products of cleavage of our multivalent minigene construct and have relied on CTL assays and/or T-cell hybridoma activation to identify appropriate presentation of the class I-restricted epitopes. None of the epitopes are flanked by their normal amino acids, instead being surrounded by sequences encoding foreign epitopes, yet most of them are presented, indicating that flanking sequences can have minimal effect on epitope processing and presentation. This is a somewhat controversial question, with some evidence suggesting that flanking sequences may exert significant effects (10, 16), while other thorough studies suggest that, at least for some epitopes, the epitopes may be processed and presented regardless of the nature of the amino acids immediately flanking them (8, 18, 26, 31). Our previous studies have failed to document any role of flanking sequences in epitope presentation; essentially all of the minigene constructs that we have made have been recognized as expected (35, 38, 39). Recent

FIG. 7. CTL specific to RSV M2₈₂₋₉₀ and to LCMV GP1₃₃₋₄₃ are induced in vivo by VV-sv. (A and B) Anti-RSV CTL. Seven days after immunization of BALB/cByJ mice with VV-sv, VV-SC11, or LCMV, splenic cells were obtained, pooled (from three mice per group), and used as effector cells in an in vitro cytotoxicity assay. BALB/C17 target cells (*H-2^d*) were coated with RSV peptide (A) or LCMV NP peptide (B). (C and D) Anti-LCMV CTL. C57BL/6 mice (eight per group) were immunized with VV-sv, VV-SC11, LCMV, or saline and 6 weeks later were challenged with LCMV. Four days later, spleens from all mice were taken and bisected. For each vaccine group, the half-spleens were pooled, homogenized, and used as effector cells in an in vitro cytotoxicity assay. As a positive effector control, cells from an LCMV-infected mouse were taken 7 days postinfection (lcmv-7). The effector (E) groups are shown on the x axis. Target (T) cells were MC57 (C) or BALB/C17 (D). Target cells were either untreated (black bars), coated with LCMV NP/L^d peptide (open bars) or LCMV GP1₃₃₋₄₃/D^b peptide (grey bars), or infected with LCMV (hatched bars).

results from other laboratories confirm that several CTL epitopes can be coexpressed in a string-of-beads array, with retention of antigen presentation despite abnormal flanking sequences (30, 31).

There is at least one alternate pathway whereby class I epitopes may be introduced into the ER; proteins targeted for cell surface expression and/or secretion are synthesized on ribosomes on the rough ER and are extruded directly into the ER, where they may undergo degradation and introduction into the class I pathway (19, 34). However, our short protein contains no known signal sequence, and this method of entry into the ER appears unlikely. It is reasonable to suppose that all three class I-restricted epitopes are processed by the standard pathway. Other studies have suggested that, for a given protein, it is the fate of approximately 3 of every 100 molecules to pass through the degradative process and to end as part of

the class I-peptide complex on the cell surface (32). Furthermore, the proteasome, or proteins participating in the proteasome complex, may be rate limiting in antigen presentation and thus may determine the immunogenicity of a given protein (15, 17). Thus, while our study relies on minigenes linked in a fusion protein, perhaps individually translated minigenes (each with its own ATG) may present an opportunity for enhanced immunogenicity, since the translation products may be able to bypass the proteasome complex and thus may experience enhanced introduction into the class I MHC pathway.

Our results establish that, in addition to CTL epitopes, T_h epitopes can be encoded in a short protein and synthesized in recombinant vaccinia virus-infected cells, successfully reaching the MHC class II antigen presentation pathway. In general, epitopes presented by class II MHC proteins are derived from lysosomal degradation of exogenous proteins taken up by spe-

FIG. 8. VV-sv immunization protects against LCMV and mengovirus challenge. (A and B) Protection against LCMV challenge. The remaining halves of the spleens taken from mice described in the legend to Fig. 7 were individually titrated in an LCMV plaque assay to determine the protective effect, if any, of VV-sv immunization. Results for all mice (eight/group) are shown as LCMV PFU/gram of spleen (A). The average PFU/gram for each group was calculated (B) and compared with the value for the saline-treated group by Student's *t* test; *P* values are shown above the groups' average values. (C) Protection against mengovirus challenge. Mice were immunized with VV-SC11 or VV-sv (10⁷ PFU i.p.) or with mengovirus (10² PFU i.p.) and were subsequently challenged with mengovirus (5 × 10⁴ PFU i.p.). At days 4 and 10 postchallenge, mice were sacrificed and the virus titers in the brains were measured. Results are presented for each mouse, and the group means are shown. A total of 39 mice were analyzed. Group sizes at each time point for VV-SC11, VV-sv, and mengovirus were as follows: on day 4, *n* = 9, 10, and 4, respectively; on day 10, *n* = 4, 6, and 6, respectively.

cialized antigen-presenting cells. However, in contrast to the relatively rigid requirements for entry into the MHC class I pathway, there appears to be a significant degree of crossover which allows endogenously synthesized protein to enter the MHC class II antigen presentation pathway (3, 6). We show here that the VSV GP₄₁₅₋₄₃₃ and MTB P₃₅₀₋₃₆₉ epitopes, presented by the I-A^b and I-A^d molecules, respectively, are expressed in a form recognizable to T-cell hybridomas and that the latter epitope can induce immunity in mice. We have not

attempted to determine whether these sequences have reached the class II molecules by crossover within the infected cell or whether they have been released from dead or dying cells and have been taken up as protein fragments by specialized antigen-presenting cells, thereby entering the traditional exogenous route to class II presentation.

The presence of several immunodominant epitopes, some presented by the same MHC alleles, raised the possibility that competition might occur, resulting in diminished responses to

one or other epitope. Peptide competition for class I binding has been suggested as a means whereby viruses might escape the attention of CTL, by generating a sequence which is poorly immunogenic but which outcompetes other, more immunogenic peptides (5). In our multivalent minigene, one possibility exists for such competition; the LCMV GP epitope is presented the class *I-D^b* allele, and the SV NP epitope, although presented mainly by *K^b*, also binds to *D^b* with high affinity (11). However, the LCMV epitope is recognized in tissue culture (Fig. 3) and induces CTL in vivo (Fig. 7), which protect against LCMV challenge (Fig. 8). Thus, competition for class I binding, if it occurs, appears biologically insignificant. The apparent failure of the promiscuous MTB epitope sequence to be presented on the *H-2^b* background may also result from its being outcompeted by the VSV peptide, presented by the same MHC allele (*I-A^b*). However, in our hands, the T-cell hybridoma was unable even to detect the appropriate peptide on the surface of *H-2^b* splenocytes. Therefore, the negative results obtained with VV-sv on the *H-2^b* background for this epitope are of somewhat questionable significance.

At first sight, the results on the *H-2^d* background for the promiscuous epitope are difficult to reconcile. T-cell hybridoma BC.H20 recognizes peptide-coated target cells but is unable to recognize splenocytes infected with VV-sv (Fig. 4C and D); however, VV-sv infection of BALB/c mice leads to priming of T cells which proliferate in response to the promiscuous peptide. At least two explanations suggest themselves. First, the correct sequence may be presented in VV-sv-infected cells at a density too low to allow recognition by BC.H20 but sufficient to induce T-cell responses in vivo. Alternatively, VV-sv may present a peptide derived from the appropriate promiscuous sequence but different from the promiscuous peptide itself (P₃₅₀₋₃₆₉). Thus, hybridoma BC.H20 is unable to recognize VV-sv-infected cells; nevertheless, T cells induced by this differently displayed variant of the promiscuous sequence are able to cross-react with the full-length peptide (P₃₅₀₋₃₆₉). This unilateral or unidirectional cross-recognition has been previously observed by us, leading us to suggest that T-cell epitopes represent a hierarchy, in which "good" epitopes induce T cells capable of recognizing the "good" sequence as well as subtle variants thereof, while T cells induced by one of the subtle variants may be unable to recognize any of the other epitopes, including the good epitope (40).

This study is the first to show that an antibody epitope embedded in a multivalent minigene construct is immunogenic and contrasts with a recent study in which antibody responses were not detected (30). In our study, the B-cell epitope was associated with two T_H epitopes; such an association may enhance the antibody response (23). It is unclear whether the mengovirus sequence is taken up by B cells as part of an intact minigene fusion protein or as an oligopeptide fragment following protein degradation. Mengovirus-specific responses were found in all six VV-sv-immunized mice, and the antibodies had virus neutralizing activity in tissue culture; furthermore, the antibodies appeared to function in vivo, since VV-sv-immunized mice exhibited markedly increased resistance to mengovirus challenge, showing a 3- to 5-log reduction in virus titers following virus challenge (Fig. 8C).

Recent work on the establishment of T-cell memory has indicated that the frequency of memory T cells specific for one virus may be reduced by subsequent infection with a different virus (28), suggesting that serial infections by different viruses may result in a constantly diminishing immunity to the preceding viruses. Our multiple minigene system provides an excellent opportunity to determine whether simultaneous priming to a variety of different epitopes (T_H, CTL, and antibody and

epitopes from several different microbes) will result in similar levels of precursor cells for all epitopes. Importantly, we will also be able to determine the effect of serial infection, either by the same or different viruses, on the effective immunological memory against each of these epitopes.

In this report, we show that the expression of multiple immunodominant epitopes results in the induction of several classes of immune response in vivo. There is minimal evidence that flanking sequences are important or that competition occurs for introduction into the MHC presentation pathways. Thus, the immune repertoire appears easily able to react simultaneously to all of these normally dominant immunogens, providing biologically relevant immune responses, including CTL which confer protection against subsequent LCMV challenge and protective neutralizing antibodies to mengovirus. This study therefore reveals the feasibility of using minigenes in designing recombinant multivalent vaccines against infectious diseases and possibly cancer.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI-27028.

We thank the numerous scientific colleagues cited in the text who contributed materials.

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